Investigating the role of MlaA in SDS-EDTA resistance pathways in *Escherichia coli*

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SUMMARY The outer membrane (OM) in Gram-negative bacteria, like *Escherichia coli,* is asymmetric, with mainly lipopolysaccharide (LPS) molecules in the outer leaflet and phospholipids in the inner leaflet. Maintaining structural asymmetry of the OM is vital for cell survival in unfavourable conditions. The Mla pathway is a mechanism that restores membrane asymmetry upon exposure to external stressors, such as sodium dodecyl sulfate (SDS) and ethylenediaminetetraacetic acid (EDTA). Deletion of the OM porin OmpC, which forms a complex with MlaA, a component of the Mla pathway, results in hypersensitivity to SDS-EDTA treatment. However, these mutants are rescued when grown in phosphate deficient conditions. The phosphoporin PhoE, which is upregulated under phosphate limitation, likely complements OmpC for maintaining OM asymmetry. PhoE shares structural and functional similarities to OmpC, but its interaction with MlaA remains unclear. Thus, we sought to investigate the role of MlaA in the OmpC- and putative PhoE-dependent pathways of restoring membrane asymmetry. We hypothesized that since OmpC and PhoE appear to mediate independent pathways for maintaining membrane asymmetry that converge on MlaA, deletion of MlaA would cause severely reduced cell growth compared to both *ΔompC* and *ΔphoE* mutants. To test this, we performed growth curve assays with wild type, *ΔompC, ΔphoE,* and *ΔmlaA* strains in minimal media containing SDS and increasing concentrations of EDTA. We found that growth of *ΔmlaA* mutants was not as severely inhibited as expected, and that *ΔompC* mutants were more sensitive to SDS-EDTA than *ΔmlaA* mutants, demonstrating the importance of MlaA-independent pathways of SDS-EDTA resistance.

INTRODUCTION

The asymmetric outer membrane (OM) of the Gram-negative bacteria, including *Escherichia coli*, is largely composed of lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet. Due to its physical and chemical properties, densely packed LPS in the outer leaflet contributes to the hydrophobicity and low permeability of the OM (1). In addition, negative charges carried by the LPS molecules are bridged and stabilized by divalent cations such as Mg^{2+} and Ca^{2+} on the OM surface, which further enhances the integrity of the OM (2). Therefore, the fortified OM can protect cells from membrane perturbation caused by reagents such as detergents (3).

The integrity of the OM can be dampened by disrupting the ionic bonding between LPS and divalent cations (2). Ethylenediaminetetraacetic acid (EDTA) is a chelating agent that removes divalent ions from the OM, creating electrostatic repulsion between LPS molecules. This destabilizes the interaction between LPS molecules in the OM, leading to the structural impairment of the OM via the loss of LPS molecules (4). To compensate for the absence of LPS, phospholipids from the inner leaflet are translocated to the outer leaflet. Due to the fluidic property of lipids, the accumulation of phospholipids at the outer leaflet increases the permeability of the OM, making the cells more susceptible to detergents like sodium dodecyl sulfate (SDS) (5). SDS molecules are structurally different from phospholipids as they are composed of a single fatty acid chain and a hydrophilic functional group. As a result, they are able to penetrate through membrane bilayers and remove phospholipids by forming micelles, leading to cell membrane solubilization (6, 7).

The OmpC-Mla system restores the asymmetry of the OM via retrograde phospholipid transport (1). The system is composed of the porin OmpC and the components of the Mla

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pathway that span the OM. OmpC is involved in passive diffusion of small molecules across the OM, and its expression is induced by high osmolarity (8). The Mla pathway is composed of the MlaA lipoprotein in the OM, MlaC in the periplasm, and the MlaFEDB complex in the inner membrane. Only MlaA, which forms a hydrophilic channel hypothesized to shuttle phospholipids, has been identified to form a complex with OmpC through direct interactions (1). When phospholipids accumulate in the outer leaflet, the OmpC-MlaA complex facilitates asymmetry restoration by transferring mislocalized phospholipids to the chaperone protein, MlaC. MlaC shuttles the transferred phospholipids through the periplasm and delivers them to the MlaFEDB complex of the inner membrane, thereby recovering membrane integrity (3). The shuttling of mislocalized phospholipids is crucial for maintaining structural asymmetry and therefore important for cell survival.

Defects in OM asymmetry restoration can be assessed by SDS-EDTA assays. Cells are incubated with SDS and EDTA and growth rates of the cells are measured. If OM asymmetry is maintained, the cell growth will be resistant to this treatment. Naturally, OmpC-deficient mutants are hypersensitive to SDS-EDTA (9). However, in a previous study by Boen *et al.*, *ΔompC* mutants regain resistance to SDS-EDTA treatment under phosphate deficient conditions. They suggested that PhoE is responsible for this phenotype as it is part of the Pho regulon, and its expression is inducible through phosphate limitation (10). Furthermore, PhoE shares functional and structural similarities to OmpC (11). Both porins have similar molecular weights (40.3 kDa and 38.9 kDa for OmpC and PhoE, respectively) and pore diameters (1.3 nm and 1.2 nm for OmpC and PhoE, respectively), and 61% of the amino acids between OmpC and PhoE are conserved (11). This leads to the possibility that PhoE may perform an analogous role in restoring membrane integrity in the absence of OmpC via an alternative pathway. Therefore, the close homology between PhoE and OmpC suggests that both porins potentially interact with MlaA. It remains to be investigated whether the Mla pathway is indeed necessary for this proposed PhoE-involved mechanism of SDS-EDTA resistance.

In this paper, we aim to investigate the role of MlaA in the OmpC- and putative PhoE-dependent pathways of membrane asymmetry restoration in sufficient (PS) and phosphate deficient (PD) conditions. In addition, given that the function of MlaA in both pathways has not been assessed under different expression levels of OmpC, which is induced under high osmolarity, we introduced the third condition - high salt (HS) to achieve this goal (8). Since MlaA forms a channel in the OM that likely facilitates phospholipid translocation, we hypothesized that the absence of MlaA would result in severely reduced cell growth in all conditions compared to both *ΔompC* and *ΔphoE* mutants (1). To test our hypothesis, we performed and analyzed SDS-EDTA growth curve assays on the wild type, *ΔompC*, *ΔphoE*, and *ΔmlaA* strains in PS, PD and HS media. Our data suggest that MlaA does not serve as a critical junction between OmpC-dependent and putative PhoE-dependent pathways, and that OmpC seems to more deeply impact asymmetry restoration in *E. coli*.

METHODS AND MATERIALS

Bacterial strains and growth conditions. *E. coli* K12 strain BW25113 (hereafter referred to as wild type), JW2203-1 (*ΔompC*) and JW0231-1 (*ΔphoE*) from the CGSC list of Keio strains were obtained from the MICB421 laboratory stock. JW2343-1 (*ΔmlaA*), also from the Keio collection, was kindly provided by Dr. Chng of the National University of Singapore. The wild type strain was grown on Lysogeny Broth (LB) Agar plates, and the mutant strains were grown on LB Agar plates supplemented with 50 μg/mL kanamycin. All strains were incubated at 37°C.

Genotype verification of *E. coli* wild type, *ΔompC, ΔphoE* and *ΔmlaA* strains. Primers for *ompC* and *phoE* genes were obtained from a previous study by Boen *et al.* and flanked the respective genes by at least 100bp (10). Primers for *ΔmlaA* mutant verification were also designed 100 bp upstream and downstream of the *mlaA* gene using the *E. coli* BW25113 genomic sequence obtained from NCBI (Table 1) (12). An alternative set of primers were used to amplify the *ompC* gene as a PCR control; these primers were also obtained from Boen *et al.* (10).

TABLE. 1 Nucleotide sequences of primers used for genotype verification PCR reactions. The *ompC* and *phoE* primer sets were acquired from Boen *et al*. and flank the respective *ompC* and *phoE* genes by at least 100bp (10). The *mlaA* primer set was designed to flank the *mlaA* gene by 100bp as well. The PCR control primer set, also sourced from Boen *et al*., amplified the *ompC* gene in the wild type strain (10).

Primer		Sequence (5'→ 3')
PCR control	Forward	GCAAATAAAGGCATATAACAGAGGGTTAATAACATG
	Reverse	CAGGCCCTTTGTTCGATATCAATCGAGATTA
ompC	Forward	GAGAATGGACTTGCCGACTGATTAATGAG
	Reverse	CACGCCAGAAGGTACCCATAGTTTTG
phoE	Forward	GATATCAAACGAACGTTTTAGCAGGACTGTCGTCGGTTG
	Reverse	GAGCTGGAAGCGCAGGAATCCCGTTTTAC
mlaA	Forward	TTACGTCTAGGTCATTGTCGG
	Reverse	CTGTTACAATCGCCCACACC

Isolation of genomic DNA from all strains was performed using Invitrogen™ PureLink™ Genomic DNA Mini Kit (Cat#K182001). Each PCR reaction contained 50 ng of genomic DNA, 0.2 µM of forward and reverse primers, 0.2 mM of dNTP mix, 1.5 mM $MgCl_2$, and 2 units/reaction Taq polymerase (Cat#10966018) in a final volume of 50 µL. The PCR reactions performed in the BioRad T100™ Thermal Cycler were initiated with denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing for 30 seconds, and extension at 72°C for 3 minutes, and ended with a final extension at 72°C for 10 minutes. Annealing temperatures for the PCR control, *ompC*, *phoE*, and *mlaA* primers were 54°C, 59°C, 64°C, and 54°C, respectively.

PCR products were loaded onto a 1.5% agarose gel containing SYBR® Safe DNA Gel Stain (Cat#S33102) in 1xTAE buffer and visualized using the BioRad ChemiDoc™ MP Imaging System. PCR products were purified using Invitrogen™ PureLink™ PCR Purification Kit (Catalog#K310002) and analyzed on the NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific) for DNA concentration and purity. Subsequently, purified PCR samples along with one of the PCR primers used in each amplification reaction were sent to Genewiz for Sanger sequencing. Sequence reads were aligned to the wild type genome and pKD13 sequence (containing the kanamycin cassette) using NCBI nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Minimal Media. The minimal media were prepared as described by Boen *et al.* (10). The base minimal media contained final concentrations of 0.12 M HEPES, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 3 mM Na_2SO_4, 1 mM $MgCl_2 \cdot 6H_2O$, 0.2 mM $CaCl_2 \cdot 2H_2O$, 3.3 µM FeCl3, 0.1 M glucose, and 3.8 µM thiamine. PD and PS media were obtained by adding K_2HPO_4 to a final concentration of 42 µM or 660 µM, respectively. HS media was obtained by adding NaCl and K_2HPO_4 to a final concentration of 0.24 M and 660 µM, respectively. HEPES buffer was adjusted with 1 M HCl to obtain a pH of 7.42. All stock solutions were dissolved separately in autoclaved distilled water and filter-sterilized using a 0.20 µm pore filter before combining. All media and media components were wrapped in aluminum foil and stored at 4°C.

LB Growth Curve. *E. coli* wild type, *ΔompC*, *ΔphoE*, and *ΔmlaA* were grown overnight in LB media at 37°C in a shaking incubator. OD_{600} readings were obtained and used to prepare seed cultures of 1×10^5 cells/mL for the wild type, *ΔompC*, *ΔphoE,* and *ΔmlaA* strains. These were added to a 96-well plate in triplicate to a final volume of 200 µL in LB. The 96-well

plate was incubated at 37°C in a BioTek Plate Reader and OD_{600} readings were obtained every 30 minutes for 16 hours, shaking for 10 seconds before each reading.

SDS-EDTA assay. A stock solution of 100 mM EDTA was dissolved, adjusted to pH 8.0, and filter-sterilized through a 0.20 μM pore filter. 1% w/v stock solution of SDS was dissolved and filter-sterilized. Overnight cultures of *E. coli* wild type, *ΔompC*, *ΔphoE*, and *ΔmlaA* strains were grown in PD, PS, and HS media for 48 hours in a shaking incubator at 37°C. Cell concentrations were calculated from OD_{600} measurements, and cells from each culture condition were added to a 96-well plate to a final concentration of 5×10^6 cells/ml, in triplicate. Each well contained the appropriate minimal media, supplemented with 0.05% w/v SDS and final concentrations of either 0 mM, 0.4 mM, or 0.8 mM EDTA in a final volume of 220 μL. The 96-well plate was incubated at 37°C in a BioTek Plate Reader and OD_{600} readings were obtained every 30 minutes for 36 hours, shaking for 10 seconds before each reading.

RESULTS

***E. coli* BW25113 mutant strains *ΔompC* (JW2203-1), *ΔphoE* (JW0231-1), and *ΔmlaA* (JW2343-1) carry deletions expected in each genotype.** We performed PCR analysis to confirm the genotypic identity of the deletion mutants (*ΔompC*, *ΔphoE*, and *ΔmlaA*) that we intended to use for our assays. Primers for amplification in *ΔompC* and *ΔphoE* mutants were sourced from Boen *et al*., which flank the respective coding regions by at least 100 base pairs (10). Primers for the *ΔmlaA* mutant were designed to have a similar flanking distance from the *mlaA* coding region (Table 1). As a positive PCR control, primers from Boen *et al.* were used to amplify the *ompC* gene in the wild type strain (10). Given that the construct used in the production of the deletion mutant strains contains a kanamycin resistance marker, we expected our PCR reactions to yield products bearing the kanamycin resistance cassette sequence. Considering the lengths of the kanamycin resistance cassette (1303 bp) and the *ompC* gene (1104 bp), we expected PCR products to have lengths of PCR Control (1165 bp), *ΔompC* (1671 bp), *ΔphoE* (1850 bp), and *ΔmlaA* (1400 bp). However, we failed to produce a functional agarose gel to resolve our bands and ladders, and were unable to confidently identify the sizes of our amplified products (Fig. S1). Thus, we compared the relative sizes of each band to confirm the correct amplification of the desired genes. The amplified products from the *ΔphoE* mutant had the largest bands, followed by *ΔompC* mutants, then *ΔmlaA* mutants, which corresponds with their expected sizes. In order to further verify the genotype of each strain, purified PCR products were sent to Genewiz for Sanger sequencing, and the results were analyzed using BLAST alignment (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Our results confirmed that each mutant contained the expected kanamycin resistance cassette insertion.

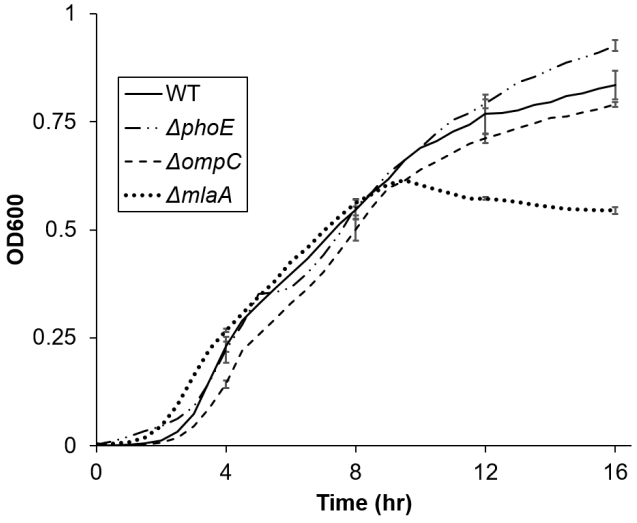

FIG. 1 Growth of *E. coli ΔompC* and *ΔphoE* strains in LB media indicate no major growth impairment, while *ΔmlaA* may experience inhibited growth in comparison to wild type. 96-well plates were seeded with initial inocula of $1x10^5$ cells/ml (*ΔompC*, *ΔphoE*) or 5×10^6 cells/mL (*ΔmlaA*) from overnight cultures of each strain, and incubated at 37°C for 16 hours. OD_{600} measurements were taken every 30 minutes. Error bars represent standard deviation of technical replicates performed for each strain and are thus not indicative of statistical significance (n=1).

***E. coli* BW25113 mutant strains *ΔompC* and *ΔphoE* exhibit no major growth defects in LB broth, but *ΔmlaA* growth appears to be reduced.** To ensure the mutant strains did not have growth defects that would influence the results of our SDS-EDTA assay, we compared the untreated growth of *ΔompC*, *ΔphoE*, and *ΔmlaA* mutants to the wild type strain in LB. We generated LB growth curves for all strains over a period of 16 hours and found that all showed comparable growth in the first 8 hours of incubation (Fig. 1). Whereas *ΔompC* and *ΔphoE* strains continued to exhibit a growth pattern similar to wild type until the end of the assay, the *ΔmlaA* strain already progressed into stationary phase beyond this point. We propose that variation in growth may be due to the fact that we tested the *ΔmlaA* strain on a separate plate using a higher initial inoculum. The greater cell density might result in greater waste accumulation, leading to an earlier stationary phase, as we observed. As such, it is likely that the *ΔmlaA* mutant might yield a growth curve comparable to those of the other strains if a consistent starting inoculum was used. Altogether, these data suggest that while *ΔompC* and *ΔphoE* mutants have similar growth patterns to the wild type strain, *ΔmlaA* mutants may suffer from a growth defect that could impact the results of our SDS-EDTA assay.

Verification of restored SDS-EDTA resistance of *ΔompC* mutants in PD conditions. Previous investigations by Boen *et al.* found that SDS resistance is restored in *ΔompC* mutants in phosphate-limiting conditions (10). To confirm these observations, we performed an SDS-EDTA assay to examine the sensitivity of the *ΔompC* strain to SDS-EDTA stress in PS and PD media. We observed a difference in the overall levels of growth between the two conditions that mirrored the data from the previous study (10). With no EDTA treatment, the wild type strain grew to an optical density of 0.3 in PS media and to a maximum of 0.2 in PD media (Fig. 2). The decreased growth in the latter condition is likely due to the growth restriction imposed by limited phosphate in the environment, and not as a result of SDS-EDTA treatment. Subsequently, we focused on the relative growth of the *ΔompC* strain in comparison to the wild type in each condition to achieve a better analysis of the resistance conferred by phosphate starvation. Consistent with previous observations, we found that in PS media, *ΔompC* mutant growth is noticeably diminished in comparison

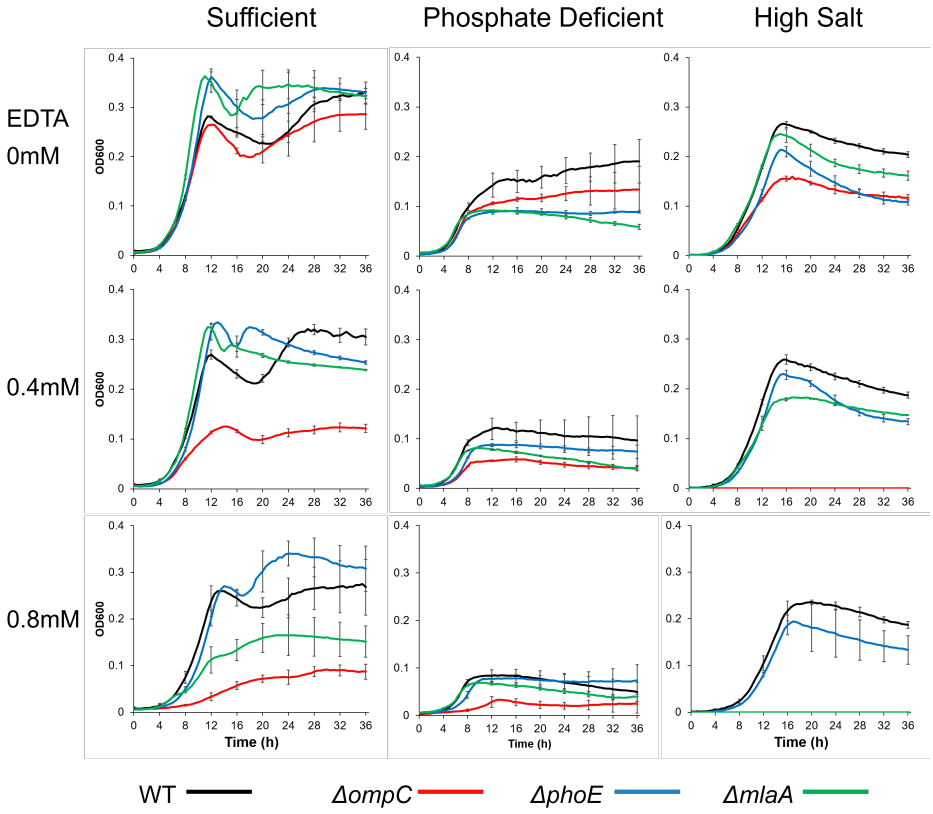

FIG. 2 Growth curves of *E. coli ΔompC*, *ΔphoE*, and *ΔmlaA* strains in PS, PD, and HS media, with supplementation of SDS and EDTA. 96-well plates containing 0.05% SDS with 0 mM, 0.4 mM, or 0.8 mM EDTA were inoculated with 48-hour overnight cultures of each strain, at a starting concentration of $5x10^6$ cells/ml. The plates were incubated for 36 hr at 37°C, with OD_{600} readings taken every 30 minutes. Error bars represent standard deviation of technical replicates performed for each strain and are thus not indicative of statistical significance (n=1).

to the wild type strain, but becomes comparable to wild type when grown in PD media (Fig. 2) (10). Specifically, at 0.4 mM EDTA the relative growth yield of the *ΔompC* strain, measured as the ratio of *ΔompC* strain optical density to wild type optical density at endpoint, is not significantly different between PS and PD conditions (Fig. 3). However, at 0.8mM EDTA, the relative growth of the *ΔompC* strain increases to 0.8 in PD media, while the relative growth decreases to less than 0.5 in PS media, indicating that *ΔompC* mutant gained resistance to SDS-EDTA treatment via phosphate limitation. We therefore successfully recapitulated the results of Boen *et al.* and demonstrated a new threshold to which SDS-EDTA resistance occurs in PD conditions (10).

MlaA does not appear to be the most essential component of OmpC- and putative PhoE-dependent pathways. We conducted SDS-EDTA assays to investigate the role of MlaA in OmpC- and putative PhoE-dependent pathways of membrane restoration. Given our hypothesis that MlaA is involved in both of these pathways, we expected the *ΔmlaA* mutant to have a severer growth defect in SDS-EDTA assays compared to the *ΔphoE* and *ΔompC* strains. Contrary to our expectations, the *ΔmlaA* strain did not exhibit severe growth impairment in comparison to *ΔompC* and *ΔphoE* strains (Fig. 2). In both PS and PD conditions, treatment of *ΔmlaA* mutants with 0.4 mM EDTA did not result in decreased growth, which is consistent with the observation that *ΔmlaA* mutants are sensitive to EDTA at concentrations of 0.8 mM and above (11). However, when this threshold concentration was met, the *ΔmlaA* strain exhibited a growth defect only in the PS condition, and to a lower degree of severity than expected. The growth of *ΔmlaA* mutants was lower than that of the wild type strain, but was still present at a greater density than *ΔompC* mutants. These data suggest that MlaA is not as essential to the OmpC-dependent mechanism of restoring OM asymmetry as we hypothesized.

Furthermore, under phosphate deficiency at the same concentration of EDTA, *ΔmlaA* mutants appear to grow unperturbed. Treatment of the *ΔmlaA* strain with 0.8 mM EDTA in PD media resulted in a growth pattern that closely mimicked the wild type strain. The lack of reduction in the growth of *ΔmlaA* mutants seems to indicate that the phosphate starvation-dependent mechanism of OM asymmetry maintenance is independent of MlaA and may provide a protective function against OM disruption that can negate the effects of MlaA absence. It is interesting to note that our results provide evidence against the role of PhoE as the primary component mediating asymmetry restoration in PD conditions. Across the range of EDTA concentrations tested, *ΔphoE* mutants consistently displayed no growth defects in PD media. This is in contrast to our prediction that the growth of *ΔphoE* mutants would be negatively impacted, which is based on the model by Boen *et al.* that induction of

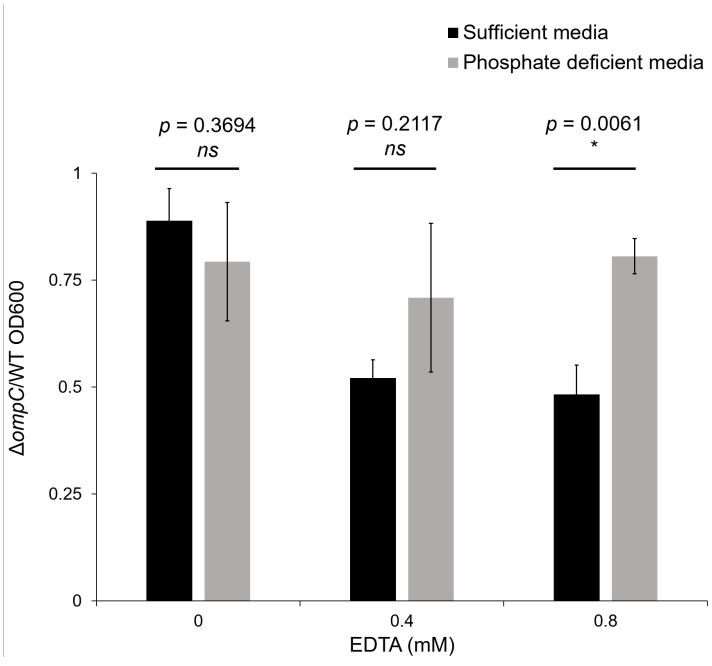

FIG. 3 *E. coli ΔompC* mutant exhibited increased resistance against SDS-EDTA under PD conditions. Relative growth of *ΔompC* strain was calculated as relative to wild type OD_{600} values after 36 hours of treatment with 0.05% SDS and increasing concentrations of EDTA in PS or PD media (data from Fig. 2 transformed). Error bars reflect standard deviation of three technical replicates of one biological replicate (n=1). Statistical analyses were performed using unpaired, two-tailed t-test (*ns* = not significant). P-value cut-off of <0.05.

PhoE is a necessary event in one of the pathways for repairing OM damage (10). However, it should be noted that OmpC is constitutively expressed in *ΔphoE* mutants, which might mask the impairment of PhoE-dependent OM asymmetry restoration.

High salt conditions did not contribute to SDS-EDTA resistance in all strains. To test the robustness of the OmpC-Mla pathway model, we sought to modify a parameter of this model, specifically OmpC levels. To this end, we repeated the SDS-EDTA assay in PS media with supplementation of 3-fold more NaCl to create a HS condition, as it has been previously established that *ompC* expression is upregulated under conditions of high osmolarity (13). We found that both wild type and *ΔphoE* mutant growth were reduced in response to the HS media. Even with no EDTA treatment, the OD_{600} values of both strains decreased by at least 0.1 in comparison to the PS conditions. Moreover, the HS media appeared to exacerbate the effects of SDS-EDTA treatment in the *ΔompC* and *ΔmlaA* mutants. Growth of *ΔompC* and *ΔmlaA* strains were completely suppressed at 0.4 mM and 0.8 mM EDTA, respectively. In comparison, growth of these strains were hindered but not fully inhibited in the PS condition at the same EDTA concentrations. These observations suggest that inducing *ompC* expression did not improve the restoration of OM asymmetry.

DISCUSSION

The structural asymmetry of the OM in Gram-negative bacteria is vital to resisting detergents, antibiotics, and other external stressors (3). Upon the loss of LPS in the outer leaflet of the OM, the phospholipids from the inner leaflet fill in the gaps in the outer leaflet, decreasing the overall asymmetry of the OM (4). The Mla system that Gram-negative bacteria use to restore this asymmetry involves the help of the OM porin, OmpC. The direct interaction between OmpC and MlaA, a component of the Mla system, has been illustrated to transport mislocalized phospholipids from the outer leaflet to downstream molecules for relocation (3). *ΔompC* strains show increased sensitivity to SDS-EDTA treatments, in which EDTA removes LPS from the OM, and SDS disrupts exposed phospholipids in the outer leaflet. However, phosphate starvation partially restores SDS-EDTA resistance in these mutants. It was speculated that this is due to a structurally similar OM protein, PhoE, being expressed in phosphate limiting conditions (10). However, the interaction between PhoE and MlaA has not yet been documented. Thus, we aimed to investigate the role of MlaA in the OmpC- and putative PhoE- dependent pathways in restoring OM asymmetry in *E. coli.* We hypothesized that mutants deficient in MlaA would have a greater growth impairment compared to *ΔompC* and *ΔphoE* mutants in SDS-EDTA growth assays because we expect MlaA to directly interact with OmpC and PhoE, forming a complex passing phospholipids to downstream molecules.

We first verified the viability of our strains in LB medium before proceeding to the SDS-EDTA assay. The LB growth curves showed that *ΔompC* and *ΔphoE* strains have no obvious growth defects, but the *ΔmlaA* strain exhibited a premature plateau (Fig. 1). We suspect that this was due to experimental error, as wild type, *ΔompC*, and *ΔphoE* strains were grown with a starting inoculum of 1×10^5 cells/mL, whereas the *ΔmlaA* strain used an initial inoculum of 5×10^6 cells/mL. The greater cell number potentially led to the accumulation of bacterial waste products, causing the culture to transition into the stationary phase earlier than expected. We as well did not see any drastic growth defects in the *ΔmlaA* strain in our downstream SDS-EDTA assays compared to wild type in the 0 mM EDTA treatments, further supporting the notion that the growth of the *ΔmlaA* mutant was likely inhibited due to an error in our LB growth assay.

After confirming strain viability, we examined the sensitivity of our mutants to membrane perturbation by performing SDS-EDTA assays. Surprisingly, the *ΔmlaA* mutant exhibited less vulnerability to the treatment than the *ΔompC* mutant. A possible explanation to this phenotype is that *E. coli* has alternative pathways with roles redundant to the Mla pathway, which compensate for the deletion of MlaA in the mutants. Studies have demonstrated the ability to restore structural asymmetry in *E. coli* via transport pathways independent of MlaA (14, 15). Two candidate operons, *yebST* and *pqiABC*, were shown to be capable of restoring membrane integrity in *E. coli*. In addition to a *ΔmlaD* background, the deletion of homologous operons *yebST* and *pqiABC* resulted in increased SDS-EDTA

sensitivity. Complementation with a *pqiBC*-expressing plasmid was able to rescue the hypersensitive phenotype, indicating the ability of this operon to restore membrane integrity. Moreover, the *yebST* operon was reportedly upregulated under deficiency in LPS production and EDTA treatment (14). This phenomenon provides promising evidence that the activity of at least one alternative pathway might be increased during our SDS-EDTA growth assays, which contributes to restoration of membrane integrity and the relatively mild impairment of *ΔmlaA* mutant growth that we observed.

Additionally, the Tol-Pal system is an alternative system to maintain OM integrity which spans the cell envelope and is proposed to be responsible for retrograde phospholipid transport (16). This system is composed of five proteins, forming two complexes including TolB-Pal in the OM and TolQRA in the cytoplasmic membrane. Their interaction and functionality have not been well elucidated. However, like the *ΔmlaA* strain, mutants in the Tol-Pal pathway exhibit disrupted OM asymmetry and have impaired retrograde phospholipid transport. Interestingly, overexpression of the Mla pathway components only partially mitigates the effects of disrupting the Tol-Pal complex (15). This lends support to the concept that besides the OmpC-Mla pathway, there exist other, perhaps more important mechanisms for restoring OM asymmetry, which may explain our finding that *ΔmlaA* mutants withstands SDS-EDTA treatment to a greater degree than *ΔompC* mutants.

Furthermore, the unexpectedly lower sensitivity of the *ΔmlaA* mutant to SDS-EDTA treatment relative to the *ΔompC* mutant aligns with evidence that OmpC plays a more integral and broad role in OM integrity than MlaA. A previous study by Chong *et al.* suggests that OmpC is involved in other mechanisms of SDS-EDTA resistance independent of OM asymmetry restoration (17). They found a comparable degree of outer leaflet phospholipid accumulation in both *ΔmlaA* and *ΔompC* strains, despite the latter exhibiting greater sensitivity to SDS-EDTA treatment. They also observed that the *ΔmlaA* strain, but not the *ΔompC* strain, is rescued from SDS-EDTA sensitivity when phospholipid accumulation is resolved through overexpression of a phospholipase that eliminates phospholipids in the outer leaflet (17). These findings indicate that OmpC may be a component of membrane repair mechanisms independent of the Mla pathway. Indeed, OmpC appears to be important in maintaining cell viability through its dynamics with OmpF. The *ΔompCΔompF* double mutant is more resistant to SDS-EDTA treatments than the *ΔompCΔompF* mutant complemented with an *ompF*-expressing plasmid (18). Therefore, OmpF confers susceptibility to SDS-EDTA stress while OmpC protects against this stress, suggesting that the maintenance of OM integrity by OmpC can occur through means independent of the Mla pathway.

In HS conditions, all strains showed a decrease in growth compared to the PS condition. In particular, *ΔompC* and *ΔmlaA* strains displayed an appreciably reduced SDS-EDTA resistance (Fig. 2). These results contrasted with our expectations that inducing *ompC* expression in the wild type and *ΔphoE* strains would result in increased cell density, as enhanced OmpC-Mla system activity might increase restoration of phospholipid mislocalization. Given our previous assumption that the OmpC-Mla system is the major mediator of OM asymmetry, we as well expected *ompC* overexpression to have no effect on the growth of *ΔompC* and *ΔmlaA* mutants in comparison to PS conditions (19). We think that by increasing the salt concentration, we consequently increased the membrane stress exerted on the cells, leading to an overall reduction in cell density. Beyond their optimal external osmolarity, *E. coli* experience a decrease in their growth density, presumably due to difficulties in regulating turgor pressure and adjusting the periplasmic osmolarity to equal that of the cytoplasm (20, 21). Thus, changes in the osmotic pressure, in combination with phospholipid accumulation in the outer leaflet, may have contributed to the enhanced susceptibility of *ΔmlaA* and *ΔompC* mutants to SDS.

Limitations We recognize that there were some limitations to our study. While growth in LB media was assessed to determine whether our strains displayed any growth defects, it does not serve as an appropriate baseline measurement for our SDS-EDTA assay. Ideally, we would have gauged the growth of our strains in each of our minimal media to obtain a better understanding of baseline growth patterns. As well, our growth assay results were merely observational, and significance in our data could not be affirmed due to our lack of

biological replicates. Thus our data may be an inaccurate portrayal of the actual growth patterns.

Furthermore, the expression levels of OmpC and PhoE were not detected directly under different conditions. Even though previous research demonstrated these two porins could be induced by high osmolarity and phosphate deficiency, respectively, it is necessary to confirm the protein expression by western blotting or RT-qPCR. This is especially important for OmpC, as previous studies have indicated that multiple mechanisms contribute towards regulating its expression (22). Thus by directly confirming the presence of these proteins, we will have more confidence in constructing a model for restoration of OM asymmetry.

Conclusions Our experiment sought to elucidate the role of MlaA in the OmpC- and putative PhoE- dependent mechanisms of OM asymmetry restoration. From our assays, we concluded that MlaA appears to be less important than OmpC in either OmpC-dependent or PD-dependent pathways. Investigating the growth of *ΔompC* and *ΔmlaA* strains under high osmolarity conditions revealed elevated sensitivity to SDS-EDTA. Overall, our study highlights the importance of MlaA-independent mechanisms of maintaining OM asymmetry in *E. coli*.

Future Directions Since studies have already been performed on the *yebST* and *pqiABC* operons in mutants with Mla pathway disruptions, further experiments can be done to explore the rescuing effect of the Tol-Pal system in *ΔmlaA* mutants. Deleting Tol-Pal proteins such as TolR has been shown to impair OM integrity (15). We suggest performing another SDS-EDTA growth assay on *ΔmlaAΔtolR* double mutants to verify whether the observed mild growth defect in the *ΔmlaA* strain is due to the presence of the Tol-Pal system, which sustains OM integrity. Alternatively, investigating the expression levels of components in the Tol-Pal pathway in *ΔmlaA* mutants may help elucidate the compensatory mechanism of this alternative pathway. RT-qPCR of *tolR* expression would provide insight on the engagement of this pathway in response to defects in the Mla pathway.

Finally, due to the strong reduction in growth of both *ΔompC* and *ΔmlaA* mutants in the HS condition, we were unable to identify finer changes in their growth as a result of increased OmpC expression. To better characterize the effects of this condition, we suggest using a lower range of EDTA concentrations, for instance 0.05 mM-0.3 mM, to account for the higher susceptibility to SDS-EDTA treatment conferred by high osmolarity.

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